TITLE

LIGANDS FOR FLT3 RECEPTORS

CROSS-REFERENCE TO RELATED APPLICATIONS

This is a continuation-in-part of United States Application 08/162,407, filed December 3, 1993, which is a continuation-in-part of United States Application 08/111,758, filed August 25, 1993, which is a continuation-in-part of United States Application 08/106,463, filed August 12, 1993, which in turn is a continuation-in-part of United States Application 08/068,394, filed May 24, 1993.

FIELD OF THE INVENTION

The present invention relates to a mammalian flt3-ligand as a homogeneous protein, the DNA encoding the ligand, pharmaceutical compositions containing the ligand, its use in marrow transplantations and in improving gene transfer therapy to mammals.

BACKGROUND OF THE INVENTION

All the blood cells originate from a common hematopoietic stem cell that becomes committed to differentiate along certain lineages, i.e., erythroid, megakaryocytic, granulocytic, monocytic, and lymphocytic. Cytokines that are known to stimulate the proliferation and maturation of various cell precursors are called "colony stimulating factors" (CSFs). Several of these CSFs are produced by T-lymphocytes and are IL-3 (multi-CSF), granulocyte-monocyte CSF (GM-CSF), granulocyte CSF (G-CSF), and monocyte CSF (M-CSF). It is known that these growth factors affect both mature cells and stem cells. Heretofore no factors have been discovered that are able to predominantly affect stem cells.

The tyrosine kinase receptors are a large family of growth factor proteins that play a role in regulating the proliferation and differentiation of a number of cells (Yarden, Y. & Ullrich, A. *Annu. Rev. Biochem.*, 57, 443-478, 1988; and Cadena, D.L. & Gill, G.N. *FASEB J.*, 6, 2332-2337, 1992). Some of the receptors function within the hematopoietic system. For example, signaling through the colony-

stimulating factor type 1 (CSF-1), receptor c-fms regulates the survival, growth and differentiation of monocytes (Stanley *et al.*, *J. Cell Biochem.*, 21, 151-159, 1983). The Steel factor (also known as mast cell growth factor, stem cell factor or kit ligand), acting through c-kit, stimulates the proliferation of cells in both the myeloid and lymphoid compartments.

Two recently described members of the tyrosine kinase receptor family, flt3 (Rosnet et al. *Oncogene*, 6, 1641-1650, 1991); and flk-2 (Matthews et al., *Cell*, 65, 1143-1152, 1991) are proteins closely related to the c-fms and c-kit receptors. The flk-2 gene product is known to be expressed on hematopoietic and progenitor cells, while the flt3 gene product has a more general tissue distribution. The flt3 and flk-2 receptor proteins are nearly identical in amino acid sequence except for a 31 amino acid segment located near the C-termini (Lyman et al., *Oncogene*, 8, 815-822, 1993). Furthermore, since the extracellular regions of both the flt3 and flk-2 receptor proteins are the same except for two amino acids (Matthews, et al., Id.; Rosnet et al., Id.), they are believed to share the same ligand. Lyman et al. further report that it is unlikely that the flt3 and flk-2 proteins are encoded by different genes given their nearly identical amino acid sequences. In addition, the characterization of the flt3 receptor is reported by Lyman et al. to be similar to the c-erbB2 protein in the flt3 receptor's ability to be phosphorylated in the absence of ligand.

The ligand for the flt3 likely would transduce self-renewal signals to regulate the growth and differentiation of progenitor and stem cells and possess clinical utility in the treatment of a number of diseases and hematopoietic disorders, and is especially useful in allogeneic, syngeneic or autologous bone marrow transplants in patients undergoing cytoreductive therapies, as well as cell expansion. The ligand also finds use in gene therapy and progenitor and stem cell mobilization systems.

With particular reference to cancer, such diseases are generally treated with various forms of cytoreductive therapies. Cytoreductive therapies involve administration of ionizing radiation or chemical toxins which are cytotoxic for rapidly dividing cells. Side effects of such therapy can be attributed to cytotoxic effects upon normal cells and can usually limit the use of cytoreductive therapies. A frequent side effect is myelosuppression, or damage to bone marrow cells which gives rise to white and red blood cells and platelets. As a result of myelosuppression, patients develop

cytopenia which are blood cell deficits. As a result of cytopenias, patients are exposed to increased risk of infection and bleeding disorders.

Cytopenia is a major factor contributing to morbidity, mortality, and under-dosing in cancer treatment. Many clinical investigators have manipulated cytoreductive therapy dosing regimens and schedules to increase dosing for cancer therapy, while limiting damage to bone marrow. One approach involves bone marrow transplantations in which bone marrow hematopoietic progenitor or stem cells are removed before a cytoreductive therapy and then reinfused following therapy to rescue bone marrow from toxicity resulting from the cytoreductive therapy. Progenitor and stem cells may implant in bone marrow and differentiate into mature blood cells to supplement reduced population of mature blood cells.

High-dose chemotherapy is therapeutically beneficial because it can produce an increased frequency of objective response in patients with metastatic cancers, particularly breast cancer, when compared to standard dose therapy. This can result in extended disease-free remission for some even poor-prognosis patients. Nevertheless, high-dose chemotherapy is toxic and many resulting clinical complications are related to infections, bleeding disorders and other effects associated with prolonged periods of myelosuppression. An excellent description of cytoreductive therapies and the usefulness of various growth factors therein is provided in U.S. Patent No. 5,199,942, which is incorporated herein by reference. Some of the growth factors that are described in the referenced patent are GM-CSF (sargramostim), a recombinant human granulocyte-macrophage colony stimulating factor, interleukin-3 (IL-3), steel factor (SF), GM-CSF/IL-3 fusion proteins, erythropoietin (EPO) and combinations thereof.

SUMMARY OF THE INVENTION

The present invention pertains to biologically active flt3-ligand (flt3-L) as a homogeneous protein. In addition, the invention is directed to an isolated DNA encoding flt3-L and to expression vectors containing cDNA encoding flt3-L. Within the scope of this invention are host cells which have been transfected or transformed with expression vectors which contain the cDNA encoding flt3-L. According to the invention, the host cells are cultured under conditions sufficient to facilitate expression of the flt3-L and the flt3-L is then recovered from the culture.

The homogeneous biologically active flt3-L can be used in pharmaceutical compositions and in methods of improving allogeneic, syngeneic or autologous transplantation. Also embodied within the scope of the invention are pharmaceutical compositions comprising flt3-L alone or in combination with other growth factors, such as interleukins, colony stimulating factors, protein tyrosine kinases and cytokines. Methods of use in improving gene therapy are also provided.

The present invention also pertains to antibodies, and in particular monoclonal antibodies, which are immunoreactive with flt3-L. Fusion proteins comprising the soluble portion of flt3-L and the constant domain of an immunoglobulin protein are also embodied in the invention.

In one aspect, the invention is directed to a method for conducting autologous hematopoietic progenitor cells or stem cell transplantation, comprising: (1) obtaining hematopoietic progenitor cells or stem cells from a patient prior to cytoreductive therapy; (2) expanding the hematopoietic progenitor cells or stem cells *ex vivo* with flt3-L alone or in conbination with an *ex vivo* growth factor selected from the group consisting of granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin-3 (IL-3), interleukin-6 (IL-6), interleukin-7 (IL-7), interleukin-11 (IL-11), interleukin-12 (IL-12), interleukin-15 (IL-15), steel factor (SF), GM-CSF/IL-3 fusion proteins, erythropoietin (EPO), granulocyte-colony stimulating factor (G-CSF) and combinations thereof, to provide a cellular preparation comprising increased numbers of hematopoietic progenitor cells or stem cells; and (3) administering the cellular preparation to the patient in conjunction with or following cytoreductive therapy. Progenitor and stem cells may be obtained from peripheral blood harvest or bone marrow explants.

The transplantation method of the invention described above optionally comprises a preliminary *in vivo* procedure comprising administering flt3-L alone or in combination with a recruitment growth factor to the patient to recruit the hematopoietic cells into peripheral blood prior to their harvest, wherein the recruitment growth factor is selected from the group consisting of GM-CSF, SF, G-CSF, EPO, IL-3, IL-6, IL-7, IL-11, IL-15, IL-12, GM-CSF/IL-3 fusion proteins, and combinations thereof.

The method of the invention described above optionally comprises a subsequent *in vivo* procedure comprising administering flt3-L alone or in combination with an engraftment growth factor to the patient following autologous transplantation of the cellular preparation to facilitate engraftment and augment proliferation of engrafted hematopoietic progenitor or stem cells from the cellular preparation. The engraftment growth factor is selected from the group consisting of GM-CSF, G-CSF, EPO, IL-3, IL-6, IL-7, IL-11, IL-12, IL-15, SF, GM-CSF/IL-3 fusion proteins and combinations thereof.

The invention further includes a progenitor or stem cell expansion media comprising cell growth media, autologous serum, and flt3-L alone or in combination with a growth factor selected from the group consisting of SF, G-CSF, EPO, IL-1, IL-3, IL-6, IL-7, IL-11, IL-12, IL-15, GM-CSF, GM-CSF/IL-3 fusion proteins, and combinations thereof.

The invention further includes the use of the flt3-L in gene therapy. The ligand permits proliferation and culturing of the early hematopoietic progenitor or stem cells that are to be transfected for use in gene therapy. Alternatively, the cDNA for the flt3-L may be transfected into cells in order to ultimately deliver its gene product to the targeted cell or tissue.

DETAILED DESCRIPTION OF THE INVENTION

The cDNA encoding the murine flt3-L has been determined and is provided in SEQ ID NO:1. The cDNA encoding the human flt3-L has been determined and is provided in SEQ ID NO:5. This discovery of the cDNAs encoding both murine and human flt3-L has provided the means to develop related inventions, i.e., expression vectors which contain the cDNAs encoding both murine and human flt3-L; host cells transfected or transformed with the expression vectors; biologically active murine and human flt3-L as homogeneous proteins; and antibodies immunoreactive with the murine and the human flt3-L. Further included in the invention are methods for performing cell transplantations, using the flt3-L of the invention, alone or in combination with other growth factors such as interleukins, colony stimulating factors, protein tyrosine kinases and cytokines. The flt3-L also finds use in improving gene therapy regimens and also as the gene product of the transfected cells used in gene therapy.

As used herein, the term "flt3-L" refers to a genus of polypeptides that bind and complex independently with flt3 and flk-2 receptors found on stem cells. The term "flt3-L" encompasses proteins having the amino acid sequence 1 to 231 of SEQ ID NO:2 or the amino acid sequence 1 to 235 of SEQ ID NO:6, as well as those proteins having a high degree of similarity or a high degree of identity with the amino acid sequence 1 to 231 of SEQ ID NO:2 or the amino acid sequence 1 to 235 of SEQ ID NO:6, and which proteins are biologically active and bind the flk-3 or the flk-2 receptor. In addition, the term refers to biologically active gene products of the DNA of SEQ ID NO:1 or SEQ ID NO:5. Further encompassed by the term "flt3-L" are the membrane-bound proteins (which include an intracellular region, a membrane region, and an extracellular region), and soluble or truncated proteins which comprise primarily the extracellular portion of the protein, retain biological activity and are capable of being secreted. Specific examples of such soluble proteins are those comprising the sequence of amino acids 28-163 of SEQ ID NO:2 and amino acids 28-160 of SEQ ID NO:6.

The term "biologically active" as it refers to flt3-L, means that the flt3-L is capable of binding to the flt3 and flk-2 receptors. Alternatively, "biologically active" means the flt3-L is capable of transducing a stimulatory signal to the cell through the membrane-bound flt3 and flk-2.

A "flt3-L variant" as referred to herein, means a polypeptide substantially homologous to native flt3-L, but which has an amino acid sequence different from that of native flt3-L (human, murine or other mammalian species) because of one or more deletions, insertions or substitutions. The variant amino acid sequence preferably is at least 80% identical to a native flt3-L amino acid sequence, most preferably at least 90% identical. The percent identity may be determined, for example, by comparing sequence information using the GAP computer program, version 6.0 described by Devereux et al. (*Nucl. Acids Res.* 12:387, 1984) and available from the University of Wisconsin Genetics Computer Group (UWGCG). The GAP program utilizes the alignment method of Needleman and Wunsch (*J. Mol. Biol.* 48:443, 1970), as revised by Smith and Waterman (*Adv. Appl. Math* 2:482, 1981). The preferred default parameters for the GAP program include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) for nucleotides, and the weighted comparison matrix of Gribskov and Burgess, *Nucl. Acids Res.* 14:6745, 1986, as described by Schwartz and Dayhoff, eds., *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, pp. 353-358, 1979; (2) a penalty of 3.0 for

each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps. Variants may comprise conservatively substituted sequences, meaning that a given amino acid residue is replaced by a residue having similar physiochemical characteristics. Examples of conservative substitutions include substitution of one aliphatic residue for another, such as Ile, Val, Leu, or Ala for one another, or substitutions of one polar residue for another, such as between Lys and Arg; Glu and Asp; or Gln and Asn. Other such conservative substitutions, for example, substitutions of entire regions having similar hydrophobicity characteristics, are well known. Naturally occurring flt3-L variants are also encompassed by the invention. Examples of such variants are proteins that result from alternative mRNA splicing events or from proteolytic cleavage of the flt3-L protein, wherein the flt3-L binding property is retained. Alternative splicing of mRNA may yield a truncated but biologically active flt3-L protein, such as a naturally occurring soluble form of the protein, for example. Variations attributable to proteolysis include, for example, differences in the N- or C-termini upon expression in different types of host cells, due to proteolytic removal of one or more terminal amino acids from the flt3-L protein (generally from 1-5 terminal amino acids).

The term "autologous transplantation" is described in U.S. Patent No. 5,199,942, which is incorporated herein by reference. Briefly, the term means a method in which bone marrow, or peripheral blood, progenitor cells or stem cells are removed from a patient prior to myelosuppressive cytoreductive therapy, expanded in *ex vivo* culture in the presence of flt3-L alone or with an additional growth factor, and then readministered to the patient concurrent with or following cytoreductive therapy to counteract the myelosuppressive effects of such therapy. In particular, the method according to the invention comprises (a) removing the hematopoietic progenitor or stem cells from the patient prior to cytoreductive therapy; (b) expanding the hematopoietic progenitor cells or stem cells *ex vivo* with flt3-ligand alone or in combination with a growth factor selected from the group consisting of an interleukin, a colony-stimulating factor and a cytokine, and combinations thereof to provide a cellular preparation comprising an expanded population of such hematopoietic progenitor cells or stem cells; and (c) administering the cellular preparation to the patient following cytoreductive therapy. Examples of such factors include: GM-CSF, SF, G-CSF, EPO, IL-3, IL-6, IL-7, IL-11, IL-15, IL-12, GM-CSF/IL-3 fusion proteins, and combinations thereof. The flt3-L is also useful in the same way for syngeneic or allogeneic transplantations. The term "allogeneic transplanation" means a method in

which bone marrow or peripheral blood progenitor cells or stem cells are removed from a mammal and administered to a different mammal of the same species. The term "syngeneic transplantation" means the bone marrow transplantation between gentically identical mammals.

The transplantation method of the invention described above optionally comprises a preliminary *in vivo* procedure comprising administering flt3-L alone or in combination with a recruitment growth factor to the patient to recruit the hematopoietic cells into peripheral blood prior to their harvest, wherein the recruitment growth factor is selected from the group consisting of GM-CSF, SF, G-CSF, EPO, IL-3, IL-6, IL-7, IL-11, IL-15, IL-12, GM-CSF/IL-3 fusion proteins, and combinations thereof.

The method of the invention described above optionally comprises a subsequent *in vivo* procedure comprising administering flt3-L alone or in combination with an engraftment growth factor to the patient following autologous transplantation of the cellular preparation to facilitate engraftment and augment proliferation of engrafted hematopoietic progenitor or stem cells from the cellular preparation. The engraftment growth factor is selected from the group consisting of GM-CSF, G-CSF, EPO, IL-3, IL-6, IL-7, IL-11, IL-12, IL-15, SF, GM-CSF/IL-3 fusion proteins and combinations thereof.

The invention further includes a progenitor or stem cell expansion media comprising cell growth media, autologous serum, and flt3-L alone or in combination with a growth factor selected from the group consisting of SF, G-CSF, EPO, IL-1, IL-3, IL-6, IL-7, IL-11, IL-12, IL-15, GM-CSF, GM-CSF/IL-3 fusion proteins, and combinations thereof.

The invention further includes the use of the flt3-L in gene therapy. The ligand permits proliferation and culturing of the early hematopoietic progenitor or stem cells that are to be transfected for use in gene therapy.

The flt3 proteins can be used to screen clones and candidate cell lines for expression of flt3-L proteins. A novel flt3:Fc fusion protein was constructed and employed in the screening procedure described below in Example 3. The flt3:Fc fusion protein offers the advantage of being easily purified. In addition, disulfide bonds form between the Fc regions of two separate fusion protein chains, creating dimers. The

dimeric flt3:Fc receptor was chosen for the potential advantage of higher affinity binding of the flt3-L, in view of the possibility that the ligand being sought would be multimeric.

Example 1 describes the construction of the flt3:Fc fusion protein utilized in the screening for the flt3-L. Other antibody Fc regions may be substituted for the human IgG1 Fc region described in Example 1. Other suitable Fc regions are those that can bind with high affinity to protein A or protein G, and include the Fc region of human IgG1 or fragments of the human or murine IgG1 Fc region, e.g., fragments comprising at least the hinge region so that interchain disulfide bonds will form.

As described supra., an aspect of the invention is soluble flt3-L polypeptides. Soluble flt3-L polypeptides comprise all or part of the extracellular domain of a native flt3-L but lack the transmembrane region that would cause retention of the polypeptide on a cell membrane. Soluble flt3-L polypeptides advantageously comprise the native (or a heterologous) signal peptide when initially synthesized to promote secretion, but the signal peptide is cleaved upon secretion of flt3-L from the cell. The soluble flt3-L polypeptides encompassed by the invention retain the ability to bind the flt3 and flk-2 receptors. Indeed, soluble flt3-L may also include part of the transmembrane region or part of the cytoplasmic domain or other sequences, provided that the soluble flt3-L protein can be secreted.

Soluble flt3-L may be identified (and distinguished from its non-soluble membrane-bound counterparts) by separating intact cells which express the desired protein from the culture medium, e.g., by centrifugation, and assaying the medium (supernatant) for the presence of the desired protein. The presence of flt3-L in the medium indicates that the protein was secreted from the cells and thus is a soluble form of the desired protein. It is possible that soluble flt3-L may be a naturally-occurring form of this protein.

Soluble forms of flt3-L possess many advantages over the native bound flt3-L protein. Purification of the proteins from recombinant host cells is feasible, since the soluble proteins are secreted from the cells. Further, soluble proteins are generally more suitable for intravenous administration.

Examples of soluble flt3-L polypeptides include those comprising the entire extracellular domain of a native flt3-L protein. Such soluble mammalian flt3-L proteins comprise amino acids 28 through 188 of SEQ ID NO:2 or amino acids 28 through 182 of SEQ ID NO:6. In addition, soluble flt-3-L proteins comprising less than the entire extracellular domain are included in the invention. Such truncated soluble proteins are represented by the sequence of amino acids 28-163 of SEQ ID NO:2, and amino acids 28-160 of SEQ ID NO:6. When initially expressed within a host cell, the soluble protein may additionally comprise one of the heterologous signal peptides described below that is functional within the host cells employed. Alternatively, the protein may comprise the native signal peptide, such that the mammalian flt3-L comprises amino acids 1 through 188 of SEQ ID NO:2 or amino acids 1 through 182 of SEQ ID NO:6. In one embodiment of the invention, soluble flt3-L is initially expressed as a fusion protein which comprises (from N- to C-terminus) the yeast α factor signal peptide, the FLAG® peptide described below and in U.S. Patent No. 5,011,912, and soluble flt3-L comprising amino acids 28 to 188 of SEQ ID NO:2. This recombinant fusion protein is expressed in and secreted from yeast cells. The FLAG® peptide facilitates purification of the protein, and subsequently may be cleaved from the soluble flt3-L using bovine mucosal enterokinase. Isolated DNA sequences encoding soluble flt3-L proteins are encompassed by the invention.

Truncated flt3-L, including soluble polypeptides, may be prepared by any of a number of conventional techniques. A desired DNA sequence may be chemically synthesized using techniques known per se. DNA fragments also may be produced by restriction endonuclease digestion of a full length cloned DNA sequence, and isolated by electrophoresis on agarose gels. Linkers containing restriction endonuclease cleavage site(s) may be employed to insert the desired DNA fragment into an expression vector, or the fragment may be digested at cleavage sites naturally present therein. The well known polymerase chain reaction procedure also may be employed to amplify a DNA sequence encoding a desired protein fragment. As a further alternative, known mutagenesis techniques may be employed to insert a stop codon at a desired point, e.g., immediately downstream of the codon for the last amino acid of the extracellular domain.

In another approach, enzymatic treatment (e.g., using *Bal* 31 exonuclease) may be employed to delete terminal nucleotides from a DNA fragment to obtain a fragment having a particular desired terminus. Among the commercially available linkers are

those that can be ligated to the blunt ends produced by *Bal* 31 digestion, and which contain restriction endonuclease cleavage site(s). Alternatively, oligonucleotides that reconstruct the N- or C-terminus of a DNA fragment to a desired point may be synthesized and ligated to the DNA fragment. The synthesized oligonucleotide may contain a restriction endonuclease cleavage site upstream of the desired coding sequence and position an initiation codon (ATG) at the N-terminus of the coding sequence.

As stated above, the invention provides homogeneous flt3-L polypeptides, both recombinant and non-recombinant. Variants and derivatives of native flt3-L proteins that retain the desired biological activity (e.g., the ability to bind flt3) may be obtained by mutations of nucleotide sequences coding for native flt3-L polypeptides. Alterations of the native amino acid sequence may be accomplished by any of a number of conventional methods. Mutations can be introduced at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites enabling ligation to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes an analog having the desired amino acid insertion, substitution, or deletion.

Alternatively, oligonucleotide-directed site-specific mutagenesis procedures can be employed to provide an altered gene wherein predetermined codons can be altered by substitution, deletion or insertion. Exemplary methods of making the alterations set forth above are disclosed by Walder et al. (*Gene* 42:133, 1986); Bauer et al. (*Gene* 37:73, 1985); Craik (*BioTechniques*, January 1985, 12-19); Smith et al. (*Genetic Engineering: Principles and Methods*, Plenum Press, 1981); Kunkel (*Proc. Natl. Acad. Sci. USA* 82:488, 1985); Kunkel et al. (*Methods in Enzymol.* 154:367, 1987); and U.S. Patent Nos. 4,518,584 and 4,737,462 all of which are incorporated by reference.

flt3 Ligands may be modified to create flt3-L derivatives by forming covalent or aggregative conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like. Covalent derivatives of flt3-L may be prepared by linking the chemical moieties to functional groups on flt3-L amino acid side chains or at the N-terminus or C-terminus of a flt3-L polypeptide or the extracellular domain thereof. Other derivatives of flt3-L within the scope of this invention include covalent or aggregative conjugates of flt3-L or its fragments with other proteins or polypeptides, such as by synthesis in recombinant culture as N-terminal or C-terminal fusions. For example, the conjugate may comprise a signal or leader polypeptide sequence (e.g. the

α-factor leader of *Saccharomyces*) at the N-terminus of a flt3-L polypeptide. The signal or leader peptide co-translationally or post-translationally directs transfer of the conjugate from its site of synthesis to a site inside or outside of the cell membrane or cell wall.

flt3 Ligand polypeptide fusions can comprise peptides added to facilitate purification and identification of flt3-L. Such peptides include, for example, poly-His or the antigenic identification peptides described in U.S. Patent No. 5,011,912 and in Hopp et al., *Bio/Technology* 6:1204, 1988. One such peptide is the FLAG® peptide, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (DYKDDDDK), which is highly antigenic and provides an epitope reversibly bound by a specific monoclonal antibody enabling rapid assay and facile purification of expressed recombinant protein. This sequence is also specifically cleaved by bovine mucosal enterokinase at the residue immediately following the Asp-Lys pairing. Fusion proteins capped with this peptide may also be resistant to intracellular degradation in *E. coli*. A murine hybridoma designated 4E11 produces a monoclonal antibody that binds the peptide DYKDDDDK in the presence of certain divalent metal cations (as described in U.S. Patent 5,011,912, hereby incorporated by reference) and has been deposited with the American Type Culture Collection under accession no. HB 9259.

The invention further includes flt3-L polypeptides with or without associated native-pattern glycosylation. flt3-Ligands expressed in yeast or mammalian expression systems (e.g., COS-7 cells) may be similar to or significantly different from a native flt3-L polypeptide in molecular weight and glycosylation pattern, depending upon the choice of expression system. Expression of flt3-L polypeptides in bacterial expression systems, such as *E. coli*, provides non-glycosylated molecules.

Equivalent DNA constructs that encode various additions or substitutions of amino acid residues or sequences, or deletions of terminal or internal residues or sequences not needed for biological activity or binding can be prepared. For example, N-glycosylation sites in the flt3-L extracellular domain can be modified to preclude glycosylation, allowing expression of a reduced carbohydrate analog in mammalian and yeast expression systems. N-glycosylation sites in eukaryotic polypeptides are characterized by an amino acid triplet Asn-X-Y, wherein X is any amino acid except Pro and Y is Ser or Thr. The murine and human flt3-L proteins each comprise two such triplets, at amino acids 127-129 and 152-154 of SEQ ID NO:2, and at amino acids

126-128 and 150-152 of SEQ ID NO:6, respectively. Appropriate substitutions, additions or deletions to the nucleotide sequence encoding these triplets will result in prevention of attachment of carbohydrate residues at the Asn side chain. Alteration of a single nucleotide, chosen so that Asn is replaced by a different amino acid, for example, is sufficient to inactivate an N-glycosylation site. Known procedures for inactivating N-glycosylation sites in proteins include those described in U.S. Patent 5,071,972 and EP 276,846, hereby incorporated by reference.

In another example, sequences encoding Cys residues that are not essential for biological activity can be altered to cause the Cys residues to be deleted or replaced with other amino acids, preventing formation of incorrect intramolecular disulfide bridges upon renaturation. Other equivalents are prepared by modification of adjacent dibasic amino acid residues to enhance expression in yeast systems in which KEX2 protease activity is present. EP 212,914 discloses the use of site-specific mutagenesis to inactivate KEX2 protease processing sites in a protein. KEX2 protease processing sites are inactivated by deleting, adding or substituting residues to alter Arg-Arg, Arg-Lys, and Lys-Arg pairs to eliminate the occurrence of these adjacent basic residues. Lys-Lys pairings are considerably less susceptible to KEX2 cleavage, and conversion of Arg-Lys or Lys-Arg to Lys-Lys represents a conservative and preferred approach to inactivating KEX2 sites. Both murine and human flt3-L contain two KEX2 protease processing sites at amino acids 216-217 and 217-218 of SEQ ID NO:2 and at amino acids 211-212 and 212-213 of SEQ ID NO:6, respectively.

Nucleic acid sequences within the scope of the invention include isolated DNA and RNA sequences that hybridize to the native flt3-L nucleotide sequences disclosed herein under conditions of moderate or severe stringency, and which encode biologically active flt3-L. Moderate stringency hybridization conditions refer to conditions described in, for example, Sambrook et al. *Molecular Cloning: A Laboratory Manual,* 2 ed. Vol. 1, pp. 1.101-104, Cold Spring Harbor Laboratory Press, (1989). Conditions of moderate stringency, as defined by Sambrook et al., include use of a prewashing solution of 5 X SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0) and hybridization conditions of about 55°C, 5 X SSC, overnight. Conditions of severe stringency include higher temperatures of hybridization and washing. The skilled artisan will recognize that the temperature and wash solution salt concentration may be adjusted as necessary according to factors such as the length of the probe.

Due to the known degeneracy of the genetic code wherein more than one codon can encode the same amino acid, a DNA sequence may vary from that shown in SEQ ID NO:1 and SEQ ID NO:5 and still encode an flt3-L protein having the amino acid sequence of SEQ ID NO:2 and SEQ ID NO:6, respectively. Such variant DNA sequences may result from silent mutations (e.g., occurring during PCR amplification), or may be the product of deliberate mutagenesis of a native sequence.

The invention provides equivalent isolated DNA sequences encoding biologically active flt3-L, selected from: (a) DNA derived from the coding region of a native mammalian flt3-L gene (e.g., cDNA comprising the nucleotide sequence presented in SEQ ID NO:1 or SEQ ID NO:5); (b) DNA capable of hybridization to a DNA of (a) under moderately stringent conditions and which encodes biologically active flt3-L; and (c) DNA which is degenerate as a result of the genetic code to a DNA defined in (a) or (b) and which encodes biologically active flt3-L. The flt3-L proteins encoded by such DNA equivalent sequences are encompassed by the invention.

DNA that are equivalents to the DNA sequence of SEQ ID NO:1 or SEQ ID NO:5, will hybridize under moderately stringent conditions to the native DNA sequence that encode polypeptides comprising amino acid sequences of 28-163 of SEQ ID NO:2 or 28-160 of SEQ ID NO:6. Examples of flt3-L proteins encoded by such DNA, include, but are not limited to, flt3-L fragments (soluble or membrane-bound) and flt3-L proteins comprising inactivated N-glycosylation site(s), inactivated KEX2 protease processing site(s), or conservative amino acid substitution(s), as described above. flt3-L Proteins encoded by DNA derived from other mammalian species, wherein the DNA will hybridize to the cDNA of SEQ ID NO:1 or SEQ ID NO:5, are also encompassed.

Variants possessing the requisite ability to bind flt3 and flk-2 receptors may be identified by any suitable assay. Biological activity of flt3-L may be determined, for example, by competition for binding to the ligand binding domain of flt3 and flk-2 receptors (i.e. competitive binding assays).

One type of a competitive binding assay for a flt3-L polypeptide uses a radiolabeled, soluble human flt3-L and intact cells expressing cell surface flt3 receptors. Instead of intact cells, one could substitute soluble flt3 receptors (such as a flt3:Fc fusion protein) bound to a solid phase through the interaction of a Protein A, Protein G or an antibody to the flt3 or Fc portions of the molecule, with the Fc region of the

fusion protein. Another type of competitive binding assay utilizes radiolabeled soluble flt3 receptors such as a flt3:Fc fusion protein, and intact cells expressing flt3-L. Alternatively, soluble flt3-L could be bound to a solid phase.

Competitive binding assays can be performed following conventional methodology. For example, radiolabeled flt3-L can be used to compete with a putative flt3-L homolog to assay for binding activity against surface-bound flt3 receptors. Qualitative results can be obtained by competitive autoradiographic plate binding assays, or Scatchard plots may be utilized to generate quantitative results.

Alternatively, soluble flt3-L can be bound to a solid phase such as a column chromatography matrix or a similar substrate suitable for analysis for the presence of a detectable moiety such as ^{125}I. Binding to a solid phase can be accomplished, for example, by binding an flt3-L:Fc fusion protein to a Protein A, Protein G or to an antibody against the flt3 or Fc portion of the molecule-containing matrix.

The binding characteristics of flt3-L (including variants) may also be determined using the conjugated, soluble flt3 receptors (for example, ^{125}I-flt3:Fc) in competition assays similar to those described above. In this case, however, intact cells expressing flt3 receptors, or soluble flt3 receptors bound to a solid substrate, are used to measure the extent to which a sample containing a putative flt3-L variant competes for binding with a conjugated a soluble flt3 to flt3-L.

The flt3-L of the present invention can be used in a binding assay to detect cells expressing flt3 receptors. For example, flt3-L or an extracellular domain or a fragment thereof can be conjugated to a detectable moiety such as ^{125}I. Radiolabeling with ^{125}I can be performed by any of several standard methodologies that yield a functional ^{125}I-flt3-L molecule labeled to high specific activity. Or an iodinated or biotinylated antibody against the flt3 region or the Fc region of the molecule could be used. Alternatively, another detectable moiety such as an enzyme that can catalyze a colorometric or fluorometric reaction, biotin or avidin may be used. Cells to be tested for flt3 receptor expression can be contacted with labeled flt3-L. After incubation, unbound labeled flt3-L is removed and binding is measured using the detectable moiety.

flt3-Ligand polypeptides may exist as oligomers, such as covalently-linked or non-covalently-linked dimers or trimers. Oligomers may be linked by disulfide bonds formed between cysteine residues on different flt3-L polypeptides. In one embodiment of the invention, a flt3-L dimer is created by fusing flt3-L to the Fc region of an antibody (e.g., IgG1) in a manner that does not interfere with binding of flt3-L to the flt3-ligand-binding domain. The Fc polypeptide preferably is fused to the C-terminus of a soluble flt3-L (comprising only the extracellular domain). General preparation of fusion proteins comprising heterologous polypeptides fused to various portions of antibody-derived polypeptides (including the Fc domain) has been described, e.g., by Ashkenazi et al. (*PNAS USA 88:*10535, 1991) and Byrn et al. (*Nature 344*:677, 1990), hereby incorporated by reference. A gene fusion encoding the flt3-L:Fc fusion protein is inserted into an appropriate expression vector. The flt3-L:Fc fusion proteins are allowed to assemble much like antibody molecules, whereupon interchain disulfide bonds form between Fc polypeptides, yielding divalent flt3-L. If fusion proteins are made with both heavy and light chains of an antibody, it is possible to form a flt3-L oligomer with as many as four flt3-L extracellular regions. Alternatively, one can link two soluble flt3-L domains with a peptide linker such as the $Gly_4SerGly_5Ser$ linker sequence described in United States Patent 5,073,627.

Thus, the invention provides oligomers of flt3-L extracellular domains or fragments thereof, linked by disulfide interactions, or expressed as fusion polymers with or without spacer amino acid linking groups. For example, a dimer of the flt3-L extracellular domain can be linked by an IgG Fc region linking group.

To facilitate the production or expression of the flt3-L, recombinant expression vectors containing the DNA encoding the flt3-L gene can be prepared according to the invention using well known methods. Host cells transfected or transformed with the flt3-L encoded expression vectors provide the expression system. Any suitable expression system may be employed and is determinable by the skilled artisan. The expression vectors include a flt3-L DNA sequence operably linked to suitable transcriptional or translational regulatory nucleotide sequences, such as those derived from a mammalian, microbial, viral, or insect gene. Examples of regulatory sequences include transcriptional promoters, operators, or enhancers, an mRNA ribosomal binding site, and appropriate sequences which control transcription and translation initiation and termination. Nucleotide sequences are "operably linked" when the regulatory sequence functionally relates to the flt3-L DNA sequence. Thus, a promoter

nucleotide sequence is operably linked to a flt3-L DNA sequence if the promoter nucleotide sequence controls the transcription of the flt3-L DNA sequence. The ability to replicate in the desired host cells, usually conferred by an origin of replication, and a selection gene by which transformants are identified, may additionally be incorporated into the expression vector.

In addition, sequences encoding appropriate signal peptides that are not native to the flt3-L gene can be incorporated into expression vectors. For example, a DNA sequence for a signal peptide (secretory leader) may be fused in frame to the flt3-L sequence so that the flt3-L is initially translated as a fusion protein comprising the signal peptide. A signal peptide that is functional in the intended host cells enhances extracellular secretion of the flt3-L polypeptide. The signal peptide is cleaved from the flt3-L polypeptide upon secretion of flt3-L from the cell.

Suitable host cells for expression of flt3-L polypeptides include prokaryotes, yeast or higher eukaryotic cells. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are described, for example, in Pouwels et al. *Cloning Vectors: A Laboratory Manual,* Elsevier, New York, (1985). Cell-free translation systems could also be employed to produce flt3-L polypeptides using RNAs derived from DNA constructs disclosed herein.

Prokaryotes include gram negative or gram positive organisms, for example, *E. coli* or *Bacilli*. Suitable prokaryotic host cells for transformation include, for example, *E. coli, Bacillus subtilis, Salmonella typhimurium,* and various other species within the genera *Pseudomonas, Streptomyces,* and *Staphylococcus*. In a prokaryotic host cell, such as *E. coli*, a flt3-L polypeptide may include an N-terminal methionine residue to facilitate expression of the recombinant polypeptide in the prokaryotic host cell. The N-terminal Met may be cleaved from the expressed recombinant flt3-L polypeptide.

Expression vectors for use in prokaryotic host cells generally comprise one or more phenotypic selectable marker genes. A phenotypic selectable marker gene is, for example, a gene encoding a protein that confers antibiotic resistance or that supplies an autotrophic requirement. Examples of useful expression vectors for prokaryotic host cells include those derived from commercially available plasmids such as the cloning vector pBR322 (ATCC 37017). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides simple means for identifying transformed cells. To

construct en expression vector using pBR322, an appropriate promoter and a flt3-L DNA sequence are inserted into the pBR322 vector. Other commercially available vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and pGEM1 (Promega Biotec, Madison, WI, USA).

Promoter sequences commonly used for recombinant prokaryotic host cell expression vectors include β-lactamase (penicillinase), lactose promoter system (Chang et al., *Nature 275*:615, 1978; and Goeddel et al., *Nature 281*:544, 1979), tryptophan (trp) promoter system (Goeddel et al., *Nucl. Acids Res. 8*:4057, 1980; and EP-A-36776) and tac promoter (Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, p. 412, 1982). A particularly useful prokaryotic host cell expression system employs a phage λ P_L promoter and a cI857ts thermolabile repressor sequence. Plasmid vectors available from the American Type Culture Collection which incorporate derivatives of the λ P_L promoter include plasmid pHUB2 (resident in *E. coli* strain JMB9 (ATCC 37092)) and pPLc28 (resident in *E. coli* RR1 (ATCC 53082)).

flt3-Ligands alternatively may be expressed in yeast host cells, preferably from the *Saccharomyces* genus (e.g., *S. cerevisiae*). Other genera of yeast, such as *Pichia* or *Kluyveromyces*, may also be employed. Yeast vectors will often contain an origin of replication sequence from a 2μ yeast plasmid, an autonomously replicating sequence (ARS), a promoter region, sequences for polyadenylation, sequences for transcription termination, and a selectable marker gene. Suitable promoter sequences for yeast vectors include, among others, promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem. 255*:2073, 1980) or other glycolytic enzymes (Hess et al., *J. Adv. Enzyme Reg. 7*:149, 1968;
and Holland et al., *Biochem. 17*:4900, 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Other suitable vectors and promoters for use in yeast expression are further described in Hitzeman, EPA-73,657. Another alternative is the glucose-repressible ADH2 promoter described by Russell et al. (*J. Biol. Chem.* 258:2674, 1982) and Beier et al. (*Nature* 300:724, 1982). Shuttle vectors replicable in both yeast and *E. coli* may be constructed by inserting DNA sequences from pBR322 for selection and replication in *E. coli* (Amp^r gene and origin of replication) into the above-described yeast vectors.

The yeast α-factor leader sequence may be employed to direct secretion of the flt3-L polypeptide. The α-factor leader sequence is often inserted between the promoter sequence and the structural gene sequence. See, e.g., Kurjan et al., *Cell* 30:933, 1982; Bitter et al., *Proc. Natl. Acad. Sci. USA* 81:5330, 1984; U. S. Patent 4,546,082; and EP 324,274. Other leader sequences suitable for facilitating secretion of recombinant polypeptides from yeast hosts are known to those of skill in the art. A leader sequence may be modified near its 3' end to contain one or more restriction sites. This will facilitate fusion of the leader sequence to the structural gene.

Yeast transformation protocols are known to those of skill in the art. One such protocol is described by Hinnen et al., *Proc. Natl. Acad. Sci. USA* 75:1929, 1978. The Hinnen et al. protocol selects for Trp^+ transformants in a selective medium, wherein the selective medium consists of 0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose, 10 μg/ml adenine and 20 μg/ml uracil.

Yeast host cells transformed by vectors containing ADH2 promoter sequence may be grown for inducing expression in a "rich" medium. An example of a rich medium is one consisting of 1% yeast extract, 2% peptone, and 1% glucose supplemented with 80 μg/ml adenine and 80 μg/ml uracil. Derepression of the ADH2 promoter occurs when glucose is exhausted from the medium.

Mammalian or insect host cell culture systems could also be employed to express recombinant flt3-L polypeptides. Baculovirus systems for production of heterologous proteins in insect cells are reviewed by Luckow and Summers, *Bio/Technology* 6:47 (1988). Established cell lines of mammalian origin also may be employed. Examples of suitable mammalian host cell lines include the COS-7 line of monkey kidney cells (ATCC CRL 1651) (Gluzman et al., *Cell* 23:175, 1981), L cells, C127 cells, 3T3 cells (ATCC CCL 163), Chinese hamster ovary (CHO) cells, HeLa cells, and BHK (ATCC CRL 10) cell lines, and the CV-1/EBNA-1 cell line derived from the African green monkey kidney cell line CVI (ATCC CCL 70) as described by McMahan et al. (*EMBO J.* 10: 2821, 1991).

Transcriptional and translational control sequences for mammalian host cell expression vectors may be excised from viral genomes. Commonly used promoter sequences and enhancer sequences are derived from Polyoma virus, Adenovirus 2,

Simian Virus 40 (SV40), and human cytomegalovirus. DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early and late promoter, enhancer, splice, and polyadenylation sites may be used to provide other genetic elements for expression of a structural gene sequence in a mammalian host cell. Viral early and late promoters are particularly useful because both are easily obtained from a viral genome as a fragment which may also contain a viral origin of replication (Fiers et al., *Nature* 273:113, 1978). Smaller or larger SV40 fragments may also be used, provided the approximately 250 bp sequence extending from the *Hind* III site toward the *Bgl* I site located in the SV40 viral origin of replication site is included.

Exemplary expression vectors for use in mammalian host cells can be constructed as disclosed by Okayama and Berg (*Mol. Cell. Biol.* 3:280, 1983). A useful system for stable high level expression of mammalian cDNAs in C127 murine mammary epithelial cells can be constructed substantially as described by Cosman et al. (*Mol. Immunol.* 23:935, 1986). A useful high expression vector, PMLSV N1/N4, described by Cosman et al., *Nature* 312:768, 1984 has been deposited as ATCC 39890. Additional useful mammalian expression vectors are described in EP-A-0367566, and in U.S. Patent Application Serial No. 07/701,415, filed May 16, 1991, incorporated by reference herein. The vectors may be derived from retroviruses. In place of the native signal sequence, a heterologous signal sequence may be added, such as the signal sequence for interleukin-7 (IL-7) described in United States Patent 4,965,195; the signal sequence for interleukin-2 receptor described in Cosman et al., *Nature 312*:768 (1984); the interleukin-4 signal peptide described in EP 367,566; the type I interleukin-1 receptor signal peptide described in U.S. Patent 4,968,607; and the type II interleukin-1 receptor signal peptide described in EP 460,846.

The flt3-L as a homogeneous protein according to the invention may be produced by recombinant expression systems as described above or purified from naturally occurring cells. The flt3-L is purified to substantial homogeneity, as indicated by a single protein band upon analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

One process for producing the flt3-L protein comprises culturing a host cell transformed with an expression vector comprising a DNA sequence that encodes flt3-L under conditions sufficient to promote expression of the flt3-L. The flt3-L protein is then recovered from culture medium or cell extracts, depending upon the expression

system employed. As is known to the skilled artisan, procedures for purifying a recombinant protein will vary according to such factors as the type of host cells employed and whether or not the recombinant protein is secreted into the culture medium.

For example, when expression systems that secrete the recombinant protein are employed, the culture medium first may be concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate can be applied to a purification matrix such as a gel filtration medium. Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Alternatively, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are preferred. Finally, one or more reversed-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, (e.g., silica gel having pendant methyl or other aliphatic groups) can be employed to further purify flt3-L. Some or all of the foregoing purification steps, in various combinations, are well known and can be employed to provide a substantially homogeneous recombinant protein.

It is possible to utilize an affinity column comprising the ligand binding domain of flt3 receptors to affinity-purify expressed flt3-L polypeptides. flt3-Ligand polypeptides can be removed from an affinity column using conventional techniques, e.g., in a high salt elution buffer and then dialyzed into a lower salt buffer for use or by changing pH or other components depending on the affinity matrix utilized. Alternatively, the affinity column may comprise an antibody that binds flt3-L. Example 6 describes a procedure for employing the flt3-L protein of the present invention to generate monoclonal antibodies directed against flt3-L.

Recombinant protein produced in bacterial culture is usually isolated by initial disruption of the host cells, centrifugation, extraction from cell pellets if an insoluble polypeptide, or from the supernatant fluid if a soluble polypeptide, followed by one or more concentration, salting-out, ion exchange, affinity purification or size exclusion chromatography steps. Finally, RP-HPLC can be employed for final purification

steps. Microbial cells can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

Transformed yeast host cells are preferably employed to express flt3-L as a secreted polypeptide in order to simplify purification. Secreted recombinant polypeptide from a yeast host cell fermentation can be purified by methods analogous to those disclosed by Urdal et al. (*J. Chromatog.* 296:171, 1984). Urdal et al. describe two sequential, reversed-phase HPLC steps for purification of recombinant human IL-2 on a preparative HPLC column.

Antisense or sense oligonucleotides comprising a single-stranded nucleic acid sequence (either RNA or DNA) capable of binding to a target flt3-L mRNA sequence (forming a duplex) or to the flt3-L sequence in the double-stranded DNA helix (forming a triple helix) can be made according to the invention. Antisense or sense oligonucleotides, according to the present invention, comprise a fragment of the coding region of flt3-L cDNA. Such a fragment generally comprises at least about 14 nucleotides, preferably from about 14 to about 30 nucleotides. The ability to create an antisense or a sense oligonucleotide, based upon a cDNA sequence for a given protein is described in, for example, Stein and Cohen, *Cancer Res.* 48:2659, 1988 and van der Krol et al., *BioTechniques* 6:958, 1988.

Binding of antisense or sense oligonucleotides to target nucleic acid sequences results in the formation of complexes that block translation (RNA) or transcription (DNA) by one of several means, including enhanced degradation of the duplexes, premature termination of transcription or translation, or by other means. The antisense oligonucleotides thus may be used to block expression of flt3-L proteins. Antisense or sense oligonucleotides further comprise oligonucleotides having modified sugar-phosphodiester backbones (or other sugar linkages, such as those described in WO91/06629) and wherein such sugar linkages are resistant to endogenous nucleases. Such oligonucleotides with resistant sugar linkages are stable *in vivo* (i.e., capable of resisting enzymatic degradation) but retain sequence specificity to be able to bind to target nucleotide sequences. Other examples of sense or antisense oligonucleotides include those oligonucleotides which are covalently linked to organic moieties, such as those described in WO 90/10448, and other moieties that increases affinity of the oligonucleotide for a target nucleic acid sequence, such as poly-(L-lysine). Further still, intercalating agents, such as ellipticine, and alkylating agents or metal complexes

may be attached to sense or antisense oligonucleotides to modify binding specificities of the antisense or sense oliginucleotide for the target nucleotide sequence.

Antisense or sense oligonucleotides may be introduced into a cell containing the target nucleic acid sequence by any gene transfer method, including, for example, $CaPO_4$-mediated DNA transfection, electroporation, or by using gene transfer vectors such as Epstein-Barr virus. Antisense or sense oligonucleotides are preferably introduced into a cell containing the target nucleic acid sequence by insertion of the antisense or sense oligonucleotide into a suitable retroviral vector, then contacting the cell with the retrovirus vector containing the inserted sequence, either *in vivo* or *ex vivo*. Suitable retroviral vectors include, but are not limited to, the murine retrovirus M-MuLV, N2 (a retrovirus derived from M-MuLV), or or the double copy vectors designated DCT5A, DCT5B and DCT5C (see PCT Application US 90/02656).

Sense or antisense oligonucleotides also may be introduced into a cell containing the target nucleotide sequence by formation of a conjugate with a ligand binding molecule, as described in WO 91/04753. Suitable ligand binding molecules include, but are not limited to, cell surface receptors, growth factors, other cytokines, or other ligands that bind to cell surface receptors. Preferably, conjugation of the ligand binding molecule does not substantially interfere with the ability of the ligand binding molecule to bind to its corresponding molecule or receptor, or block entry of the sense or antisense oligonucleotide or its conjugated version into the cell.

Alternatively, a sense or an antisense oligonucleotide may be introduced into a cell containing the target nucleic acid sequence by formation of an oligonucleotide-lipid complex, as described in WO 90/10448. The sense or antisense oligonucleotide-lipid complex is preferably dissociated within the cell by an endogenous lipase.

Now that the flt3-L has been cloned, and homogeneous preparations of the ligand are now possible, many uses for it become available. In essence, the flt3-L finds use in the enhancement or growth of cells expressing the flk-2 receptor or flt3 receptor, including non-hematopoietic cells. Some specifically described examples of such use for the flt3-L follow.

In particular, the invention includes a method for conducting autologous hematopoietic progenitor cell or stem cell transplantation, comprising: (1) obtaining

hematopoietic progenitor cells or stem cells from a patient prior to cytoreductive therapy; (2) expanding the hematopoietic progenitor cells or stem cells *ex vivo* with flt3-L and an additional *ex vivo* growth factor selected from the group consisting of granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin-3 (IL-3), interleukin-6 (IL-6), interleukin-7 (IL-7), interleukin-11 (IL-11), interleukin-12 (IL-12), interleukin-15 (IL-15), steel factor (SF), GM-CSF/IL-3 fusion proteins, erythropoietin (EPO), granulocyte-colony stimulating factor (G-CSF) and combinations thereof, to provide a cellular preparation comprising increased numbers of hematopoietic progenitor cells or stem cells; and (3) administering the cellular preparation to the patient in conjunction with or following cytoreductive therapy. Progenitor and stem cells may be obtained from peripheral blood harvest or bone marrow explants.

The transplantation method of the invention described above optionally comprises a preliminary *in vivo* procedure comprising administering flt3-L and a recruitment growth factor to the patient to recruit the hematopoietic cells into peripheral blood prior to their harvest, wherein recruitment growth factors are preferably selected from the group consisting of GM-CSF, SF, G-CSF, EPO, IL-3, IL-6, IL-7, IL-11, IL-12, IL-15, GM-CSF/IL-3 fusion proteins, and combinations thereof.

The method of the invention described above optionally comprises a subsequent *in vivo* procedure comprising administering flt3-L and an engraftment growth factor to the patient following autologous transplantation of the cellular preparation to facilitate engraftment and augment proliferation of engrafted hematopoietic progenitor or stem cells from the cellular preparation. Engraftment growth factors are preferably selected from the group consisting of GM-CSF, G-CSF, EPO, IL-3, IL-6, IL-7, IL-11, IL-12, IL-15, SF, GM-CSF/IL-3 fusion proteins and combinations thereof.

The flt3-L is a prime candidate for use in gene therapy due to it's expression product's specificity for progenitor and stem cells. Gene therapy has many potential uses in treating disease and has been reviewed extensively. The principle of gene therapy is based on the premise that foreign DNA-infected cells are injected into an irradiated or genetically anemic recipient host and are allowed to engraft. See e.g., Boggs, *International J. Cell Cloning*, 8:80-96, (1990); Kohn et. al., *Cancer Invest.*, 7(2):179-192 (1989); Lehn, *Bone Marrow Transpl..*, 5:287-293 (1990); and Verma, *Scientific American*, pp. 68-84 (1990). Using gene therapy methods known per se in

the art, the flt3-L cDNA of the invention is inserted into a suitable vehicle, preferably a recombinant retrovirus, together with suitable promoter and enhancer elements that permit its expression, followed by insertion into a "delivery" cell. Such a method of transferring a gene to a mammal comprises the steps of (a) culturing early hematopoietic cells in media comprising flt3-L alone or in conbination with a growth factor selected from the group consisting of SF, G-CSF, EPO, IL-1, IL-3, IL-6, IL-7, IL-11, IL-12, IL-15, GM-CSF, GM-CSF/IL-3 fusion proteins, and combinations thereof; (b) transfecting the cultured cells from step (a) with the gene; and (c) administering the transfected cells to the mammal. Within this method is the novel method of transfecting progenitor or stem cells with a gene comprising the steps of: (a) culturing such cells in media comprising flt3-L alone or in combination with a growth factor selected from the group consisting of SF, G-CSF, EPO, IL-1, IL-3, IL-6, IL-7, IL-11, IL-12, IL-15, GM-CSF, GM-CSF/IL-3 fusion proteins, and combinations thereof; and (b) transfecting the cultured cells from step (a) with the gene.

In addition to the above, the following examples are provided to illustrate particular embodiments and not to limit the scope of the invention.

EXAMPLE 1
Preparation of flt3-Receptor:Fc Fusion Protein

This example describes the cloning of murine flt3 cDNA, and the construction of an expression vector encoding a soluble murine flt3-receptor:Fc fusion protein for use in detecting cDNA clones encoding an flt3-ligand (flt3-L). Polymerase chain reaction (PCR) cloning of the flt3 cDNA from a murine T-cell was accomplished using the oligonucleotide primers and the methods as described by Lyman et al., *Oncogene*, 8:815-822, (1993), incorporated herein by reference. The cDNA sequence and encoded amino acid sequence for mouse flt3 receptor is presented by Rosnet et el., *Oncogene*, 6:1641-1650, (1991), hereby incorporated by reference. The mouse flt3 protein is reported as having a 542 amino acid extracellular domain, a 21 amino acid transmembrane domain, and a 437 amino acid cytoplasmic domain.

Prior to fusing the murine flt3 cDNA to the N-terminus of cDNA encoding the Fc portion of a human IgG1 molecule, the amplified mouse flt3 cDNA fragment was inserted into Asp718-*Not*I site of pCAV/NOT, described in PCT Application WO

90/05183. DNA encoding a single chain polypeptide comprising the Fc region of a human IgG1 antibody was cloned into the *Spe*I site of the pBLUESCRIPT SK® vector, which is commercially available from Stratagene Cloning Systems, La Jolla, California. This plasmid vector is replicable in *E. coli* and contains a polylinker segment that includes 21 unique restriction sites. A unique *Bgl*II site was introduced near the 5' end of the inserted Fc encoding sequence, such that the *Bgl*II site encompasses the codons for amino acids three and four of the Fc polypeptide.

The encoded Fc polypeptide extends from the N-terminal hinge region to the native C-terminus, i.e., is an essentially full-length antibody Fc region. Fragments of Fc regions, e.g., those that are truncated at the C-terminal end, also may be employed. The fragments preferably contain multiple cysteine residues (at least the cysteine residues in the hinge reaction) to permit interchain disulfide bonds to form between the Fc polypeptide portions of two separate flt3:Fc fusion proteins, forming dimers as discussed above.

An Asp718 restriction endonuclease cleavage site was introduced upstream of the flt3 coding region. An Asp 718-*Not*I fragment of mouse flt3 cDNA (comprising the entire extracellular domain, the transmembrane region, and a small portion of the cytoplasmic domain) was isolated. The above-described Asp718-*Not*I flt3 partial cDNA was cloned into the pBLUESCRIPT SK® vector containing the Fc cDNA, such that the flt3 cDNA is positioned upstream of the Fc cDNA. Single stranded DNA derived from the resulting gene fusion was mutagenized by the method described in Kunkel (*Proc. Natl. Acad. Sci. USA* 82:488, 1985) and Kunkel et al. (*Methods in Enzymol.* 154:367, 1987) in order to perfectly fuse the entire extracellular domain of flt3 to the Fc sequence. The mutagenized DNA was sequenced to confirm that the proper nucleotides had been removed (i.e., transmembrane region and partial cytoplasmic domain DNA was deleted) and that the flt3 and Fc sequences were in the same reading frame. The fusion cDNA was then excised and inserted into a mammalian expression vector designated sfHAV-EO 409 which was cut with *Sal*I-*Not*I, and the *Sal*I and Asp718 ends blunted. The sfHAV-EO vector (also known as pDC406) is described by McMahan et al. (*EMBO J.*, 10; No. 10: 2821-2832 (1991)).

flt3:Fc Fusion proteins preferably are synthesized in recombinant mammalian cell culture. The flt3:Fc fusion-containing expression vector was transfected into CV-1 cells (ATCC CCL 70) and COS-7 cells (ATCC CRL 1651), both derived from monkey

kidney. The flt3:Fc fusion protein expression level was relatively low in both CV-1 and COS-7 cells. Thus, expression in 293 cells (transformed primary human embryonal kidney cells, ATCC CRL 1573) was attempted.

The 293 cells transfected with the sfHAV-EO/flt3:Fc vector were cultivated in roller bottles to allow transient expression of the fusion protein, which is secreted into the culture medium *via* the flt3 signal peptide. The fusion protein was purified on protein A sepharose columns, eluted, and used to screen cells for the ability to bind the flt3:Fc protein, as described in Examples 2 and 3.

EXAMPLE 2: Screening Cells for flt3:Fc Binding

Approximately 100 different primary cells and cell lines falling into the following general categories: primary murine fetal brain cells, murine fetal liver cell lines, rat fetal brain cell lines, human lung carcinoma (fibroblastoid) cell lines, human and murine lymphoid and myeloid cell lines were assayed for flt3:Fc binding. Cell lines were incubated with flt3:Fc, followed by a biotinylated anti-human Fc antibody, followed by streptavidin-phycoerythrin (Becton Dickinson). The biotinylated antibody was purchased from Jackson Immunoresearch Laboratories. Streptavidin binds to the biotin molecule attached to the anti-human Fc antibody, which in turn binds to the Fc portion of the flt3:Fc fusion protein. Phycoerythrin is a fluorescent phycobiliprotein which serves as a detectable label. The level of fluorescence signal was measured for each cell type using a FACScan® flow cytometer (Becton Dickinson). The cell types deemed positive for flt3:Fc binding were identified.

EXAMPLE 3: Isolation and Cloning of flt3 Ligand cDNA from Murine T-Cell cDNA Library

A murine T-cell cDNA library of cell line P7B-0.3A4 was chosen as a possible source of flt3-ligand cDNA. P7B-0.3A4 is a murine T cell clone that is Thy1.2$^+$, CD4$^-$, CD8$^-$, TCR*ab*$^\pm$, CD44$^+$. It was originally cloned at a cell density of 0.33 cells/well in the presence of rHuIL-7 and immobilized anti-CD3 MAb, and was grown in continuous culture for more than 1 year by passage once a week in medium containing 15 ng/ml rHuIL-7. The parent cell line was derived from lymph node cells of SJL/J mice immunized with 50 nmoles $PLP_{139-151}$ peptide and 100 μg *Mycobacterium tuberculosis* H37Ra in Incomplete Freund's Adjuvant. PLP is the

proteolipid protein component of the myelin sheath of the central nervous system. The peptide composed of amino acids 139-151 has previously been shown to be the encephalogenic peptide in experimental autoimmune encephalomyelitis (EAE), a murine model for multiple sclerosis in SJL/J mice. (Touhy, V.K., Z. Lu, R.A. Sobel, R.A. Laursen and M.B. Lees. 1989. Identification of an encephalitogenic determinant of myelin proteolipid protein for SJL mice. *J. Immunol.* 142:1523.) After the initial culture in the presence of antigen, the parent cell line, designated PLP7, had been in continuous culture with rHuIL-7 (and without antigen) for more than 6 months prior to cloning.

P7B-0.3A4 proliferates only in response to very high concentrations of $PLP_{139-151}$ peptide in the presence of irradiated syngeneic splenocytes and is not encephalogenic or alloresponsive. This clone proliferates in response to immobilized anti-CD3 MAb, IL-2, and IL-7, but not IL-4.

Binding of flt3:Fc was observed on murine T-cells and human T-cells, and therefore a murine T-cell line was chosen (0.3A4) due to its ease of growth. A murine 0.3A4 cDNA library in sfHAV-EO was prepared as described in McMahan et al. (*EMBO J.*, 10; No:10; 2821-2832 1991). sfHAV-EO is a mammalian expression vector that also replicates in *E. coli*. sfHAV-EO contains origins of replication derived from SV40, Epstein-Barr virus and pBR322 and is a derivative of HAV-EO described by Dower et al., *J.Immunol.* 142:4314 (1989). sfHAV-EO differs from HAV-EO by the deletion of the intron present in the adenovirus 2 tripartite leader sequence in HAV-EO. Briefly, murine T-cell cDNA was cloned into the *Sal*I site of sfHAV-EO by an adaptor method similar to that described by Haymerle et al (*Nucl. Acids Res.* 14:8615, 1986), using the following oligonucleotide adapter pair:

5' TCGACTGGAACGAGACGACCTGCT 3' SEQ ID NO:3
3' GACCTTGCTCTGCTGGACGA 5' SEQ ID NO:4

Double-stranded, blunt-ended, random-primed cDNA was prepared from 0.3A4 poly (A)+ RNA essentially as described by Gubler and Hoffman, *Gene*, 25:263-269 (1983), using a Pharmacia DNA kit. The above adapters were added to the cDNA as described by Haymerle et al.. Low molecular weight material was removed by passage over Sephacryl S-1000 at 65 °C, and the cDNA was ligated into sfHAV-EO410, which had previously been cut with *Sal*I and ligated to the same oligonucleotide pair. This vector

is designated as sfHAV-EO410. DNA was electroporated (Dower et al., *Nucleic Acids Res.*, 16:6127-6145, (1988) into *E. coli* DH10B, and after one hour growth at 37 °C, the transformed cells were frozen in one milliliter aliquots in SOC medium (Hanahan et al., *J. Mol. Biol.*, 166:557-580, (1983) containing 20% glycerol. One aliquot was titered to determine the number of ampcillin-resistant colonies. The resulting 0.3A4 library had 1.84 million clones.

E. coli strain DH10B cells transfected with the cDNA library in sfHAV-EO410 were plated to provide approximately 1600 colonies per plate. Colonies were scraped from each plate, pooled, and plasmid DNA prepared from each pool. The pooled DNA, representing about 1600 colonies, was then used to transfect a sub-confluent layer of CV-1/EBNA-1 cells using DEAE-dextran followed by chloroquine treatment, similar to that described by Luthman et al., *Nucl. Acids Res.* 11:1295, (1983) and McCutchan et al., *J. Natl. Cancer Inst.* 41:351, (1986). The CV-1/EBNA-1 cell line (ATCC CRL10478) constitutively expresses EBV nuclear antigen-1 driven from the CMV immediate-early enhancer/promoter. CV1-EBNA-1 was derived from the African Green Monkey kidney cell line CV-1 (ATCC CCL 70), as described by McMahan et al. (*EMBO J.* 10:2821, 1991).

In order to transfect the CV-1/EBNA-1 cells with the cDNA library, the cells were maintained in complete medium (Dulbecco's modified Eagle's media (DMEM) containing 10% (v/v) fetal calf serum (FCS), 50 U/ml penicillin, 50 U/ml streptomycin, 2 mM L-glutamine) and were plated at a density of about 2 x 10^5 cells/well on single-well chambered slides (Lab-Tek). Slides were pretreated with 1 ml human fibronectin (10 µg/ml in PBS) for 30 minutes followed by 1 wash with PBS. Media was removed from the adherent cell layer and replaced with 1.5 ml complete medium containing 66.6 µM chloroquine sulfate. Two-tenths ml of DNA solution (2 µg DNA, 0.5 mg/ml DEAE-dextran in complete medium containing chloroquine) was then added to the cells and incubated for 5 hours. Following the incubation, the media was removed and the cells shocked by addition of complete medium containing 10% DMSO for 2.5 to 20 minutes followed by replacement of the solution with fresh complete medium. The cells were cultured for 2 to 3 days to permit transient expression of the inserted sequences.

Transfected monolayers of CV-1/EBNA-1 cells were assayed for expression of flt3-L by slide autoradiography essentially as described by Gearing et al. (*EMBO J.*

8:3667, 1989). Transfected CV-1/EBNA-1 cells (adhered to chambered slides) were washed once with binding medium with nonfat dry milk (BM-NFDM) (RPMI medium 1640 containing 25 mg/ml bovine serum albumin (BSA), 2 mg/ml sodium azide, 20 mM HEPES, pH 7.2, and 50 mg/ml nonfat dry milk). Cells were then incubated with flt3:Fc in BM-NFDM (1 μg/ml) for 1 hour at room temperature. After incubation, the cell monolayers in the chambered slides were washed three times with BM-NFDM to remove unbound flt3:Fc fusion protein and then incubated with 40 ng/ml ^{125}I-mouse anti-human Fc antibody (described below) (a 1:50 dilution) for 1 hour at room temperature. The cells were washed three times with BM-NFDM, followed by 2 washes with phosphate-buffered saline (PBS) to remove unbound ^{125}I-mouse anti-human Fc antibody. The cells were fixed by incubating for 30 minutes at room temperature in 2.5% glutaraldehyde in PBS, pH 7.3, washed twice in PBS and air dried. The chamber slides containing the cells were exposed on a Phophorimager (Molecular Dynamics) overnight, then dipped in Kodak GTNB-2 photographic emulsion (6x dilution in water) and exposed in the dark for 3-5 days at 4 °C in a light proof box. The slides were then developed for approximately 4 minutes in Kodak D19 developer (40 g/500 ml water), rinsed in water and fixed in Agfa G433C fixer. The slides were individually examined with a microscope at 25-40x magnification and positive cells expressing flt3-L were identified by the presence of autoradiographic silver grains against a light background.

The mouse anti-human Fc antibody was obtained from Jackson Laboratories. This antibody showed minimal binding to Fc proteins bound to the Fcγ receptor. The antibody was labeled using the Chloramine T method. Briefly, a Sephadex G-25 column was prepared according to the manufacturer's instructions. The column was pretreated with 10 column volumes of PBS containing 1% bovine serum albumin to reduce nonspecific adsorption of antibody to the column and resin. Nonbound bovine serum albumin was then washed from the column with 5 volumes of PBS lacking bovine serum albumin. In a microfuge tube 10 μg of antibody (dissolved in 10 μl of PBS) was added to 50 μl of 50 mM sodium phosphate buffer (pH 7.2) 2.0 mCi of carrier-free Na^{125}I was added and the solution was mixed well. 15 μl of a freshly prepared solution of chloramine-T (2 mg/ml in 0.1 M sodium phosphate buffer (pH 7.2)) was then added and the mixture was incubated for 30 minutes at room temperature, and the mixture then was immediately applied to the column of Sephadex G-25. The radiolabelled antibody was then eluted from the column by collecting 100-150 μl fractions of eluate. Bovine serum albumin was added to the eluted fractions

containing the radiolabeled antibody to a final concentration of 1%. Radioiodination yielded specific activities in the range of 5-10 x 10^{15} cpm/nmol protein.

Using the slide autoradiography approach, the approximately 1,840,000 cDNAs were screened in pools of approximately 1,600 cDNAs until assay of one transfectant pool showed multiple cells clearly positive for flt3:Fc binding. This pool was then partitioned into pools of 500 and again screened by slide autoradiography and a positive pool was identified. This pool was partitioned into pools of 100 and again screened. Individual colonies from this pool of 100 were screened until a single clone (clone #6C) was identified which directed synthesis of a surface protein with detectable flt3:Fc binding activity. This clone was isolated, and its 0.88kb cDNA insert was sequenced.

The nucleotide and encoded amino acid sequences of the coding region of the human flt3-ligand cDNA of clone 6C are presented in SEQ ID NOs:1 and 2. The cDNA insert is 0.88kb in length. The open-reading frame within this sequence could encode a protein of 231 amino acids. Thus, DNA and encoded amino acid sequences for the 231-amino acid open reading frame are presented in SEQ ID NOs:1 and 2. The protein of SEQ ID NO:2 is a type I transmembrane protein, with an N-terminal signal peptide (amino acids 1 to 27), an extracellular domain (amino acids 28-188) a transmembrane domain (amino acids 189-211) and a cytoplasmic domain (amino acids 212-231). The predicted molecular weight of the native protein following cleavage of the signal sequence is 23,164 daltons. The mature protein has an estimated pI of 9.372. There are 56 bp of 5' noncoding sequence and 126 bp of 3' non-coding sequence flanking the coding region, including the added cDNA adapters.

The vector sfHAV-EO410 containing the flt3 cDNA in *E. coli* DH10B cells was deposited with the American Type Culture Collection, Rockville, MD, USA (ATCC) on April 20, 1993 and assigned accession number ATCC 69286. The deposit was made under the terms of the Budapest Treaty.

EXAMPLE 4: Cloning of flt3-L in Yeast

For expression of soluble flt3-L in yeast, synthetic oligonucleotide primers were used to amplify via PCR (Mullis and Faloona, *Meth. Enzymol.* 155:335-350, 1987) the entire extracellular coding domain of the flt3-L between the end of the signal

peptide and the start of the transmembrane segment. The 5' primer (5'-AATTGGTACCTTTGGATAAAAGAGACTACAAG-GACGACGATGACAAGACACCTGACTGTTACTTCAGCCAC-3') SEQ ID NO:7 encoded a portion of of the alpha factor leader and an antigenic octapeptide, the FLAG sequence fused in-frame with the predicted mature N-terminus of flt3-L. The 3' oligonucleotide (5'-ATATGGATCCCTACTGCCTGGGCCGAGGCTCTGGGAG-3') SEQ ID NO:8 created a termination codon following Gln-189, just at the putative transmembrane region. The PCR-generated DNA fragment was ligated into a yeast expression vector (for expression in *K. lactis*) that directs secretion of the recombinant product into the yeast medium (Fleer et. al., *Gene*, 107:285-195 (1991); and van den Berg et. al., *Bio/Technology*, 8:135-139 (1990)). The FLAG:flt3-L fusion protein was purified from yeast broth by affinity chromotography as previously described (Hopp et. al., *Biotechnology*, 6:1204-1210, 1988).

EXAMPLE 5: Cloning of Human flt3-L

The human flt3-L was cloned from a human clone 22 T cell λgt10 random primed cDNA library as described by Sims et al., *PNAS*, 86:8946-8950 (1989). The library was screened with a 413 bp Ple I fragment from the extracellular domain of the murine flt3-L (nucleotides bp 103-516 of SEQ ID NO:1). The fragment was random primed, hybridized overnight to the library filters at 55 C in oligo prehybridization buffer. The fragment was then washed at 55 C at 2 x SSC/0.1% SDS for one hour, followed by 1 x SSC/0.1% SDS for one hour and then by 0.5 x SSC/0,1% SDS for one hour. The DNA from the positive phage plaques was extracted, and the inserts were amplified by PCR using oligonucleotides specific for the phage arms. The DNA then was sequenced, and the sequence for clone #9 is shown in SEQ ID NO:5.

The sequencing of the 988 bp cDNA clone #9 revealed an open reading frame of 705 bp surrounded by 29 bp of 5' non-coding sequence and 250 bp of 3' non-coding sequence. The 3' non-coding region did not contain a poly-A tail. There were no in-frame stop codons upstream of the initiator methionine. The open reading frame encodes a type I transmembrane protein of 235 amino acids as shown by amino acids 1-235 of SEQ ID NO:6. The protein has an N-terminal signal peptide of alternatively 26 or 27 amino acids. There exists a slightly greater probablility that the N-terminal signal peptide is 26 amino acids in length than 27 amino acids in length. The signal peptide followed by a 156 or a 155 amino acid extracellular domain (for signal peptides

of 26 and 27 amino acids, respectively); a 23 amino acid transmembrane domain and a 30 amino acid cytoplasmic domain. The human flt3-L shares overall 72% amino acid identity and 78% amino acid similarity with the murine flt3-L. The vector pBLUESCRIPT SK(-) containing the human flt3-L cDNA of clone #9 was deposited with the American Type Culture Collection, Rockville, Maryland, USA (ATCC) on August 6, 1993 and assigned acession number ATCC 69382. The deposit was made under the terms of the Budapest Treaty.

EXAMPLE 6: Monoclonal Antibodies to flt3-L

This example illustrates the preparation of monoclonal antibodies to flt3-L. flt3 Ligand is expressed in mammalian host cells such as COS-7 or CV-1/EBNA-1 cells and purified using flt3:Fc affinity chromatography. Purified flt3-L, a fragment thereof such as the extracellular domain, synthetic peptides or cells over expressing the recombinant flt3-L protein can be used to generate monoclonal antibodies against flt3-L using conventional techniques, for example, those techniques described in U.S. Patent 4,411,993. Briefly, mice are immunized with flt3-L as an immunogen emulsified in complete Freund's adjuvant, and injected in amounts ranging from 10-100 μg subcutaneously or intraperitoneally. Ten to twelve days later, the immunized animals are boosted with additional flt3-L emulsified in incomplete Freund's adjuvant. Mice are periodically boosted thereafter on a weekly to bi-weekly immunization schedule. Serum samples are periodically taken by retro-orbital bleeding or tail-tip excision to test for flt3-L antibodies by dot blot assay, ELISA (Enzyme-Linked Immunosorbent Assay) or inhibition of flt3 binding.

Following detection of an appropriate antibody titer, positive animals are provided one last intravenous injection of flt3-L in saline. Three to four days later, the animals are sacrificed, spleen cells harvested, and spleen cells are fused to a murine myeloma cell line, e.g., NS1 or preferably P3x63Ag8.653 (ATCC CRL 1580). Fusions generate hybridoma cells, which are plated in multiple microtiter plates in a HAT (hypoxanthine, aminopterin and thymidine) selective medium to inhibit proliferation of non-fused cells, myeloma hybrids, and spleen cell hybrids.

The hybridoma cells are screened by ELISA for reactivity against purified flt3-L by adaptations of the techniques disclosed in Engvall et al., *Immunochem.* 8:871, 1971

and in U.S. Patent 4,703,004. A preferred screening technique is the antibody capture technique described in Beckmann et al., (*J. Immunol.* 144:4212, 1990) Positive hybridoma cells can be injected intraperitoneally into syngeneic BALB/c mice to produce ascites containing high concentrations of anti-flt3-L-L monoclonal antibodies. Alternatively, hybridoma cells can be grown *in vitro* in flasks or roller bottles by various techniques. Monoclonal antibodies produced in mouse ascites can be purified by ammonium sulfate precipitation, followed by gel exclusion chromatography. Alternatively, affinity chromatography based upon binding of antibody to protein A or protein G can also be used, as can affinity chromatography based upon binding to flt3-L.

EXAMPLE 7: Synergistic Compositions Containing flt3-L and Either IL-7, or IL-3

This example demonstrates the synergistic stimulation of AA4.1$^+$ fetal liver cells by compositions containing flt3-L and IL-7; as well as the synergistic stimulation of c-*kit*$^+$ cells by compositions containing flt3-L and IL-3.

flt3-L and IL-7

AA4.1$^+$ expressing cells were isolated from the livers of day 14 fetal C57BL/6 mice by cell panning in Optilux 100 mm plastic Petri dishes (Falcon No. 1001, Oxnard, CA). Plates were coated overnight at 4 °C in PBS plus 0.1% fetal bovine serum (FBS) containing 10 µg/ml AA4.1 antibody (McKearn et. al., *J. Immunol.*, 132:332-339, 1984) and then washed extensively with PBS plus 1% FBS prior to use. A single cell suspension of liver cells was added at 10^7 cells/dish in PBS plus 1% FBS and allowed to adhere to the plates for two hours at 4 °C. The plates were then extensively washed, and the adhering cells were harvested by scraping. FACS analysis using AA4.1 antibody demonstrated a >95% AA4.1$^+$ cell population.

AA4.1+ Fetal liver cells were cultured in recombinant IL-7 (U.S. Patent No. 4,965,195) at 100ng/ml and recombinant yeast flt3-L at 250 ng/ml. The flt3-L was used in three different forms in the experiments: (1) as present on fixed, flt3-L-transfected CV1/EBNA cells; (2) as concentrated culture supernatants from these same flt3-L-transfected CV1/EBNA cells; and (3) as a purified and homogeneous preparation from yeast supernatant as described in Example 4.

flt3-L and IL-3

C-*kit* expressing pluripotent stem cells were purified from adult mouse bone marrow (de Vries et. al. , *J. Exp. Med.*, 176:1503-1509, 1992; and Visser and de Vries, *Methods in Cell Biol.*, 1993, submitted). Low density cells ($\leq$ 1.078 g/cm^3) positive for the lectin wheat germ agglutinin and negative for the antigens recognized by the B220 and 15-1.4.1 (Visser et. al., *Meth. in Cell Biol.*, No. 33:451-468, 1990) monoclonal antibodies, could be divided into sub-populations of cells that do and do not express c-*kit* by using biotinylated Steel factor. The c-*kit* fraction has been shown to contain the pluripotent hematopoietic stem cells (de Vries et. al., *Science* 255:989-991, 1992; Visser and de Vries, *Methods in Cell Biol.*, 1993, submitted; and Ware et. al., 1993, submitted).

Hematopoiesis Assays

The proliferation of c-*kit* $^+$ stem cells, fetal liver AA4.1$^+$ cells was assessed in [3H]-thymidine incorporation assays as described by deVries et. al., *J. Exp. Med.*, 173:1205-1211, 1991. In some experiments, 7,500 transfected CV1/EBNA cells were fixed with 1% paraformaldehyde and added to the 96-well flat-bottom tissue culture plates before the addition of cells to be assayed. CV1/EBNA cells were transfected with the flt3-L cDNA or empty expression vector pDC410. The vector pDC410 is similar to pDC406 (McMahan et al., *EMBO* J., 10; No. 10:2821-2832 (1991)) however, the EBV origin of replication of pDC406 is replaced by DNA encoding the large T antigen (driven from an SV40 promoter). The pDC410 multiple cloning site (mcs) differs from that of pDC406 in that it contains additional restriction sites and three stop codons (one in each reading frame). A T7 polymerase promoter downstream of the mcs facilitates sequencing of DNA inserted into the mcs. Purified c-*kit*$^+$ stem cells were cultured at 37 °C in a fully humidified atmosphere of 6.5% CO_2 and 7% O_2 in air for 96 hours. Murine recombinant IL-3 was used at a final concentration of 100 ng/ml. Subsequently, the cells were pulsed with 2 μCi per well of [^{3}H]-thymidine (81 Ci/mmol; Amersham Corp., Arlington Heights, IL) and incubated for an additional 24 hours. AA4.1$^+$ cells (20,000 cells/well) were incubated in IL-7, flt3-L and flt3-L + IL-7 for 48 hours, followed by [^{3}H]-thymidine pulse of six hours. The results of flt3-L and IL-7 are shown in Table I, and results of flt3-L and IL-3 are shown in Table II.

TABLE I

Effect of flt3-L and IL-7 on Proliferation of AA4.1+ Fetal Liver Cells.

	Factor			
	Control	flt3-L	IL-7	flt3-L + IL-7
[^{3}H]-thymidine incorporation (CPM)	100	1000	100	4200

The combination of flt3-L and IL-7 produced a synergistic response that was approximately four-fold greater than flt3-L alone and approximately 40-fold greater than IL-7 alone.

TABLE II

Effect of flt3-L and IL-3 on Proliferation of C-*kit*+ Cells.

	Factor			
	Control (vector alone)	flt3-L	IL-3	flt3-L + IL-3
[^{3}H]-thymidine incorporation (CPM)	100	1800	3000	9100

Culture supmatant from CV1/EBNA cells transfected with the flt3-L stimulated the proliferation of c-*kit*$^+$ stem cells approximately 18-fold greater than the culture supernatant of CV1/EBNA cells transfected with the expression vector alone. Addition of IL-3 to the flt3-L containing supernatant showed a synergistic effect, with approximately twice the number of cpm observed than would be expected if the effects were additive.

EXAMPLE 8: Construction of flt3-L:Fc Fusion Protein

This example describes the construction of a fusion protein comprising an extracellular region of the flt3-L and the Fc domain of a human immunoglobulin. The methods are essentially the same as those described in Example 1 for construction of a flt3:Fc fusion protein.

Prior to fusing the flt3-L cDNA to the N-terminus of cDNA encoding the Fc portion of a human IgG1 molecule, the flt3-L cDNA fragment is inserted into Asp718-*Not*I site of pCAV/NOT, described in PCT Application WO 90/05183. DNA encoding a single chain polypeptide comprising the Fc region of a human IgG1 antibody is cloned into the *Spe*I site of the pBLUESCRIPT SK® vector, which is commercially available from Stratagene Cloning Systems, La Jolla, California. This plasmid vector is replicable in *E. coli* and contains a polylinker segment that includes 21 unique restriction sites. A unique *Bgl*II site is then introduced near the 5' end of the inserted Fc encoding sequence, such that the *Bgl*II site encompasses the codons for amino acids three and four of the Fc polypeptide.

The encoded Fc polypeptide extends from the N-terminal hinge region to the native C-terminus, i.e., is an essentially full-length antibody Fc region. Fragments of Fc regions, e.g., those that are truncated at the C-terminal end, also may be employed. The fragments preferably contain multiple cysteine residues (at least the cysteine residues in the hinge reaction) to permit interchain disulfide bonds to form between the Fc polypeptide portions of two separate flt3-L:Fc fusion proteins, forming dimers.

An Asp718-*Stu*I partial cDNA of flt3flk-2-L in pCAV/NOT can be cloned into a Asp718-S*pe*I site of pBLUESCRIPT SK® vector containing the Fc cDNA, such that the flt3-L cDNA is positioned upstream of the Fc cDNA. Single stranded DNA derived from the resulting gene fusion can be mutagenized by the method described in Kunkel (*Proc. Natl. Acad. Sci. USA* 82:488, 1985) and Kunkel et al. (*Methods in Enzymol.* 154:367, 1987) in order to perfectly fuse the entire extracellular domain of flt3-L to the Fc sequence. The mutagenized DNA can be then sequenced to confirm that the proper nucleotides are removed (i.e., transmembrane region and partial cytoplasmic domain DNA are deleted) and that the flt3-L and Fc sequences are in the same reading frame. The fusion cDNA is then excised and inserted using conventional methods into the mammalian expression vector pCAV/NOT which is cut with Asp 718-NotI.

flt3-L:Fc Fusion proteins preferably are synthesized in recombinant mammalian cell culture. The flt3-L:Fc fusion-containing expression vector are then transfected into CV-1 cells (ATCC CCL 70) and COS-7 cells (ATCC CRL 1651), both derived from monkey kidney. Expression in 293 cells (transformed primary human embryonal kidney cells, ATCC CRL 1573) can be attempted.

The 293 cells transfected with the pCAV/NOT/flt3-L:Fc vector are cultivated in roller bottles to allow transient expression of the fusion protein, which is secreted into the culture medium *via* the flt3-L signal peptide. The fusion protein can be purified on protein A sepharose columns.

EXAMPLE 9
Activity of Truncated flt-3/flk-2-L

This example describes the preparation and activity of truncated human and murine flt-3/flk-2-L. Truncated human flt-3/flk-2-L comprising the sequence of amino acids 28-182 of SEQ ID NO:6 was prepared from yeast by following the procedure used to clone truncated murine flt3-L in yeast as described in Example 4. Two forms of truncated murine flt-3/flk-2-L comprising the sequence of amino acids 28-163 or 28-188 of SEQ ID NO:2 were each prepared from yeast following procedures as described in Example 4.

To assess the biological activity of the truncated flt-3/flk-2-L versions, it was necessary to utilize a cell line that was IL-3 dependent and that lacked the flt-3/flk-2 receptor. The BAF/BO3 cell line possesses those requirements (Hatakeyama, et. al., *Cell*, 59:837-845 (1989)). The BAF/BO3 cells were transfected with the pCAV/NOT (also known as pDC302) vector which possessed the gene for flt-3/flk-2. Briefly, BAF/BO3 (two million cells/ml) cells were electroporated with pCAV/NOT expression plasmid (Mosley et. el., *Cell*, 59:335-348 (1989)) with or without the flt3 receptor cDNA and pSV2neo cDNA (in a 10:1 ratio; the neo plasmid allows for selection in G418) in a 0.4 cm gap cuvette. Electroporations were performed at 0.3V and 960 mF using a Biorad Gene Pulser (Richmond, CA). Cells were then cultured for 24 hours in growth medium, after which flt3 expressing cells were selected for by growing the cells in soluble recombinant yeast-derived flt3-L for five days (selection in G418 was not required to obtain flt3 receptor-expressing cells). The cells were continuously cultured in RPMI 1640 plus 10% fetal bovine serum with murine IL-3 at 100 ng/ml. These cells were designated BAF/flt3.

The proliferation of the BAF/BO3 and BAF/flt3 cells was assessed in [^{3}H] TdR incorporation assays, using methods that are per se well known. Each of the BAF/BO3 and BAF/flt3 cells were seeded at 2 X 10^5 cells/ml in 50 µL/well in 96 well plates (total volume was 100 µL per well). Samples containing either murine flt3-L comprising the

sequence of amino acids 28-163 amino acids, or 28-188 amino acids; or human flt3-L comprising the sequence of amino acids 28-182 amino acids, were serially titrated 2-fold in duplicate wells. The cells were washed 2-3 times in media without IL-3. After an incubation period of approximately 40 hours, the cells were pulsed with 2 μCi per well of [^{3}H] TdR for six hours. The cells were placed in incubators with an environment of 7% CO_2, normal O_2 tension at 37 °C. All samples were harvested and counted as previously described by de Vries et. al., *J. Exp. Med.*, 173:1205-1211 (1991). Results (in approximate counts per minute CPM) of the assay are shown in Tables IIIA, IIIB, IV and V below.

TABLE IIIA

Effect of Murine flt-3/flk-2-L (28-188) on the Proliferation of BAF/BO3 Cells (CPM/1000)

Dilution	IL-3	muflt-3-L
1:2	30	0
1:4	28	0
1:8	48	0
1:20	42	0
1:40	36	0
1:80	25	0
1:200	18	0
1:400	12	0

The BAF/B03 cells did not proliferate in response to the complete extracellular domain of the murine flt-3/flk-2-L (comprising amino acids 28-188 of SEQ ID NO:2), but as expected did proliferate in response to IL-3.

TABLE IIIB

Effect of Murine flt-3/flk-2-L (28-188) on the Proliferation of BAF/flt-3 Cells (CPM/1000)

Dilution	IL-3	muflt-3-L (CV-1)	muflt3-L (yeast)
1:2	30	8	15
1:4	38	13	6
1:8	51	11	4
1:20	49	8	3
1:40	43	4	1
1:80	33	2	0
1:200	25	1	0
1:400	21	0	0

BAF/flt-3 cells proliferated in the presence of both CV1-EBNA-derived soluble (amino acids 28-188 of SEQ ID NO:2) murine flt3-L, and yeast-derived soluble (amino acids 28-188 of SEQ ID NO:2) flt3-L in a dose-dependent manner.

TABLE IV

Effect of Truncated Murine flt-3/flk-2-L on the Proliferation of BAF/flt-3 Cells (CPM/1000)

ng/ml	Background	muflt-3-L (28-163)	muflt3-L (28-188)
1:8	1	1	1
1:20	1	1	1
1:40	1	1	3
1:80	1	3	5
1:200	1	5	8
1:400	1	7	12
1:800	1	11	16
1:1000	1	16	18

A truncated version of the soluble murine flt-3/flk-2-L (comprising amino acids 28-163 of SEQ ID NO:2) possesses similar biological activity to "full length" soluble murine flt-3/flk-2-L (amino acids 28-188 of SEQ ID NO:2) on BAF/flt3 cells in a dose-dependent manner.

TABLE V

Effect of Truncated Human flt-3/flk-2-L on the Proliferation of BAF/flt-3 Cells (CPM/1000)

Dosage (ng/ml)	Background	huflt-3-L (27-182)
0.4	0.4	1
0.8	0.4	1.25
2	0.4	2.5
4	0.4	3.75
8	0.4	5.0
20	0.4	6.5
40	0.4	7.5
80	0.4	8.5
200	0.4	7.5
400	0.4	7.5
800	0.4	8.7
1000	0.4	8.5
2000	0.4	8.5

The human flt-3/flk-2-L (comprising the sequence of amino acids 27-182 of SEQ ID NO:6) also displayed biological activity on BAF/flt3 cells in a dose-dependent manner.

The BAF/B03 cells did not proliferate in response to the full length soluble murine flt-3/flk-2-L (comprising the sequence of amino acids 28-188 of SEQ ID NO:2) (Table IIIA). However, BAF/flt-3 cells proliferated in the presence of CV1-EBNA cells expressing murine flt3-L (Table IIIB). Additionally, BAF/flt3 cells proliferated in the presence of yeast-derived murine flt3-L in a dose-dependent manner (Table IIIB). The truncated version of the soluble murine flt-3/flk-2-L (comprising amino acids 28-163 of SEQ ID NO:2) possesses similar biological activity to the soluble protein on BAF/flt3 cells (Table IV). The truncated human flt-3/flk-2-L (comprising amino acids 27-182 of SEQ ID NO:6) also displayed biological activity (Table V). The BAF/flt-3 cells did not proliferate in an empty expression vector conditioned media or in yeast negative control supernatant (data not shown).